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Identification and Location of Human Papillomavirus Type 16 Antisense Early Promoter and Characterisation of Antisense RNA

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Antisense RNA sequences of various regions of human papillomavirus type 16 (HPV 16) were previously found in a number of cervical lesions, but the viral or cellular promoter has not been identified. HPV 16 E7 oncogene antisense transcripts expressed from an antisense promoter in viral DNA were found in the present study by RNase protection assays for total and cytoplasmic RNA. The antisense promoter for these transcripts was located within HPV 16 nt 4030–4230 by deletion analyses. The results also suggested that most of the antisense RNA was relatively short. The antisense promoter of HPV 16 was functional for expression of antisense RNA of a heterologous gene. Antisense-sense double-stranded E7 RNA was detected, and the sense RNA of this duplex was apparently inefficient for splicing or cleavage/poly(A) addition. These results show that HPV 16 can produce early region antisense RNA, which is from a promoter within a defined region of the viral genome. The possible importance of these transcripts for the regulation of episomal HPV 16 gene expression in infected and premalignant lesions and the possible importance of their deregulation for expression in malignant lesions are discussed. ***J. Med. Virol. 51:344–354, 1997.***

KEY WORDS: HPV 16; antisense RNA; antisense promoter; transcription; gene expression; splicing

INTRODUCTION

Human papillomaviruses (HPVs) are associated with infections of various epithelial and mucosal tissues that form benign and premalignant lesions containing mostly episomal viral DNA. HPVs are also associated with neoplastic lesions containing mostly integrated viral DNA [zur Hausen and de Villiers, 1994]. The more than 20 genital HPVs are classified into high-risk and low-risk groups based on the oncogenic severity of the lesions that they cause [Lorincz *et al.,* 1992]. Low-risk group HPVs are associated with benign lesions. In contrast, high-risk group HPVs, of which HPV 16 is the major type, are associated with high-grade premalignant and malignant lesions of the anogenital epithelia. The additional smaller role for neoplasia in nongenital sites has become apparent for specific types of HPV and nongenital cancer, such as upper respiratory tract and skin cancers [Tieben *et al.,* 1994; zur Hausen and de Villiers, 1994]. Almost all cervical carcinomas containing HPV 16 also express the E6 and E7 early region oncogenes, further implicating HPV 16 as the major aetiological agent of cervical cancer [Farthing and Vousden, 1994].

Most HPV 16 genes are initiated from the major promoter, which is termed P97 [Smotkin and Wettstein, 1986; Smotkin *et al.,* 1989]. The presence of antisense (AS) HPV 16 RNA in cervical carcinomas and carcinoma-derived cell lines was also shown. The RNA was AS to noncoding sequences and to open reading frames (ORFs) for E6-E7 and L1 [Vormwald-Dogan *et al.,* 1992]. In another report, the AS RNA represented various regions examined within the genome [Higgins *et al.,* 1991]. Attempts to map the 5′ ends of these AS transcripts had limited success because the ends were heterogeneous. This was probably due to the integrated status of the DNA since these transcripts were thought to originate from cellular flanking sequences [Vormwald-Dogan *et al.,* 1992].

AS RNA is important for gene expression, mostly because the AS RNA and mRNA form double-stranded RNA (dsRNA) [Nellen *et al.,* 1992]. dsRNA is generally unstable and defective for posttranscriptional processing, especially for pre-mRNA splicing. Therefore,

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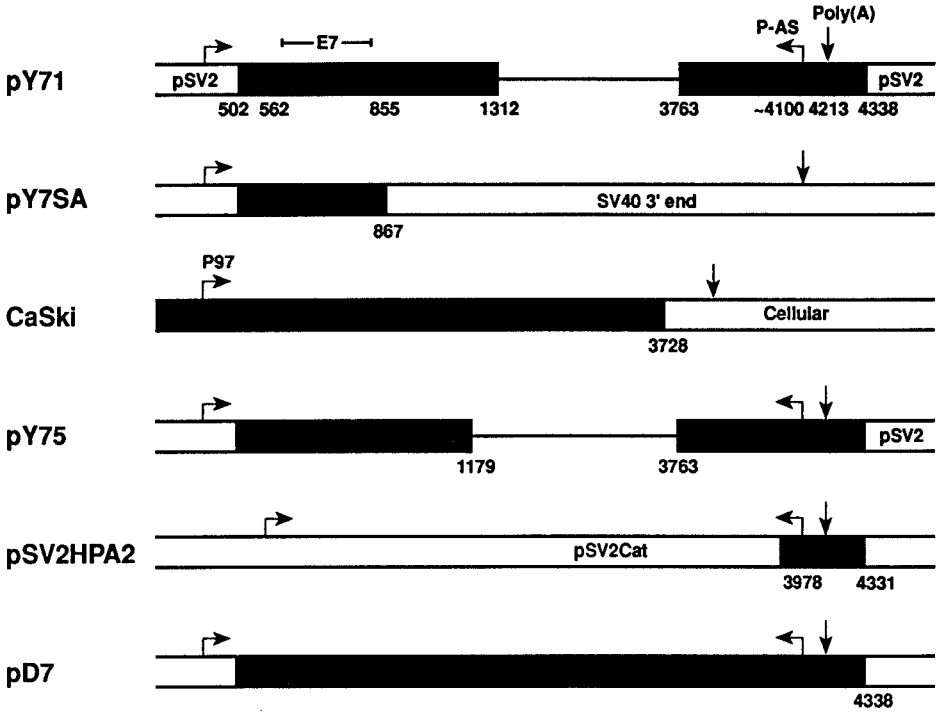

Fig. 1. Diagrams of HPV 16 DNA in plasmids and CaSki. Nt of HPV 16 positions are indicated. Black boxes indicate HPV sequences. Lines represent deleted sequences. Labelled boxes: pSV2, pSV2 expression vector sequences; SV40 3′-end, SV40 3′-end processing sequences; Cellular, CaSki cervical carcinoma cellular DNA sequences; pSV2Cat, pSV2cat plasmid CAT ORF-containing sequences. Other labels: E7, E7 ORF; P97, HPV 16 P97 early sense promoter; P-AS, antisense (AS) promoter; Poly(A), poly(A) addition site. Diagrams are not to scale.

dsRNA can be important for specific regulation of gene expression. Specific regulation also could occur for the expression of mRNA from the episomal DNA of infecting viruses. Expression of HPV 16 may be regulated in the differentiating epithelium by the dsRNA-specific unwindase that is found in such cells [Wagner *et al.,* 1989]. Thus, HPV 16 dsRNA would be important for the pattern of gene expression that is found in benign and premalignant lesions containing episomal viral DNA. In contrast, the deregulation of HPV 16 expression would be important for malignantly proliferating epithelial cells in which HPV 16 is usually integrated. In this report, AS RNA complementary to the E7 ORF and other early region sequences of HPV 16 was detected. The AS promoter was located at the 3′-end of the early region of the HPV 16 genome. Evidence was found that AS RNA inhibited the posttranscriptional processes of splicing and poly(A) addition.

MATERIALS AND METHODS

Recombinant Plasmids

HPV 16 DNA of plasmids and the CaSki cervical cell line control that was used is shown in Figure 1. The pD7 SV40 promoter-enhancer expression parental vector containing all the early region HPV 16 sequences except E6 was described earlier [Marshall *et al.,* 1989]. The pY71 SV40 vector containing E5 and E7 and parts of E6, E1, and E2 HPV 16 ORFs was also described earlier [Belaguli *et al.,* 1992]. The pD7 5′ series of deletions that originated from nt 3763 and were extended to nt 1179 for pY75 (Figs. 1–4) and to nt 1144, 1004, 954, and 876 (Fig. 4A) were also described earlier [Belaguli *et al.,* 1992]. The 3′ series of deletions from nt 1312 to nt 3896, 3971, and 4031 (Fig. 4B) were prepared as follows: *Bal*31 digestion from *Nar*I of pY71, subsequent *Pvu*I digestion, and then insertion of the smaller group of heterogeneous *Bal*31 digestion products into a second pY71 digestion's larger *Nar*I-*Pvu*I fragment. For pY7SA, which contains the SV40 vector and no HPV sequences 3′ of E7, the remaining HPV 16 sequences that were present in pY71 were inserted into the SV40 vector. The smaller of the pY71 *Hpa*II-*Nco*I HPV 16 fragments and the larger pSV2DHFR *Hind*III-*Bgl*II fragment were used. For pSV2HPA2 that was used to examine AS promoter function for the heterologous chloramphenicol acetyltransferase (CAT) gene, the smaller *Ssp*I-*Pvu*I pY71 fragment was inserted into the larger *Hpa*I-*Pvu*I pSV2cat fragment (Fig. 1).

Cell Culture and Transfection

Cells were maintained in Dulbecco's modified Eagle medium containing 10% fetal calf serum. To prepare cells for studying expression of AS RNA from episomal genomes, subconfluent COS-1 cells were transfected with 10 μg of test plasmids, with or without pRSVCat, pRSV-β-gal, and pSV2cat, and adjusted to 20 μg with pUC19. The calcium phosphate transfection method was used [Chen and Okayama, 1987]. Medium was changed after 12 hours of incubation with the transfecting DNA.

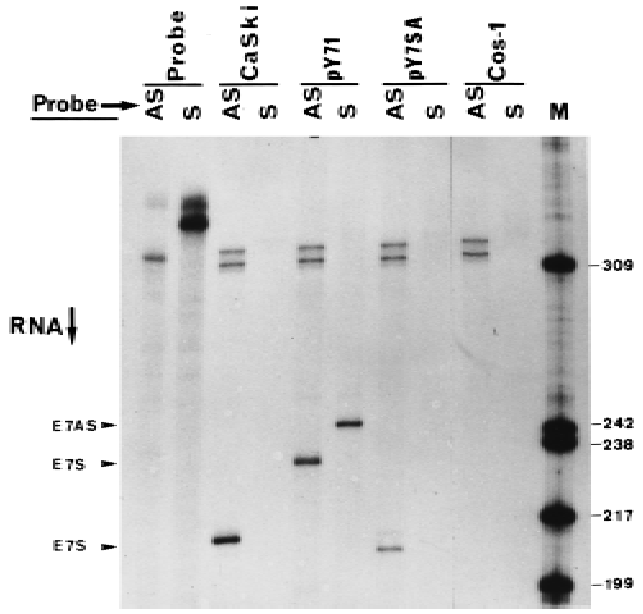

Fig. 2. E7 antisense (AS) RNA from promoter within HPV 16 DNA. RNase protection assays are shown. Labels on top of individual lanes represent the nt 502–720 E7 region of the AS RNA probe and sense (S) probe. Also labelled on top are transfected constructs and CaSki, as described in Figure 1; nontransfected cell control COS-1 (Cos-1); undigested HPV 16 E7 probes (Probe); and molecular size markers (M). Other labels indicate E7AS and E7S RNA signals on the left and marker sizes in bases on the right. Results shown represent cut sections of X-ray film because the one gel was electrophoresed with a different order of samples. The CaSki signal has a faster mobility because the AS probe also contains vector 5′ sequences present in plasmids but not CaSki. All RNase protection assays were reproducible in two to three experiments performed with at least two different preparations of RNA.

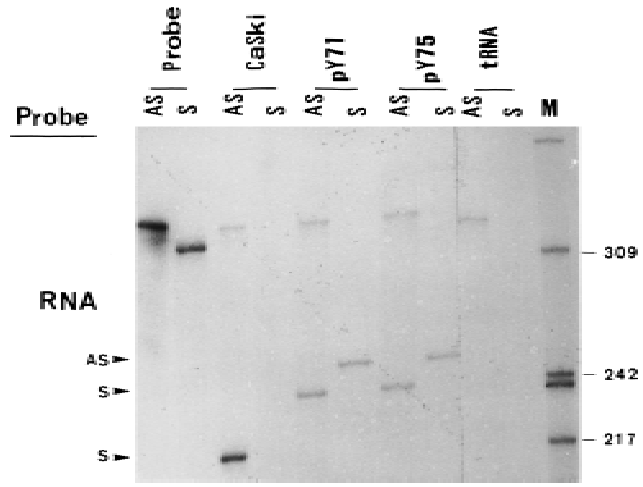

Fig. 3. Cytoplasmic HPV 16 antisense (AS) RNA. Conditions and labels are as in Figure 2. Control tRNA assay is indicated.

RNA Analysis

Total cellular RNA [Chirgwin *et al.,* 1979] and cytoplasmic RNA [Sambrook *et al.,* 1989] was isolated 48 hours after transfection. Analysis for levels of E7 AS and sense (S) RNA was by RNase protection assays. The standard RNase protection assay for total RNA was described earlier [Kreig and Melton, 1987; Belaguli *et al.,* 1992]. Briefly, this technique involves the synthesis of a uniformly labelled RNA probe. The probe is synthesised *in vitro* using either T3 or T7 polymerase to produce S or AS RNA single-strand-labelled probe from the same HPV sequence insert. The polymerase cognate phage promoter sequences are present in the BlueScript cloning vector at either end of the insert. The probe is hybridised with RNA under stringent conditions. This is followed by the removal of unhybridised single-stranded RNA by RNase digestion. RNase protection assay for dsRNA is by additional RNase digestion of single-stranded cellular RNA before the denaturation, hybridisation, and RNase digestion that is also used for standard assays. These assays for dsRNA were as described [Krystal *et al.,* 1990] and used 1,000 U of RNase T1. The probes were complementary to nt 3063–3286 for β-galactosidase internal control in pCH110 (Pharmacia, Uppsala, Sweden) and to the nt 5018–4768 CAT-coding sequences internal control of pSV2cat. All RNase protection assays were reproducible in two to three experiments carried out with at least two different preparations of RNA.

RESULTS

AS HPV 16 RNA and AS HPV 16 Promoter

Latent and active infections by HPV 11 were found to produce low levels of AS RNA, and latent infections produced no spliced E6 or E7 [Maran *et al.,* 1995]. We examined the possibility that a promoter within the genome of infecting or transfected HPV 16 directs synthesis of AS early region RNA. Therefore, transient transfection of COS-1 cells was used to produce detectable levels of RNA from an episomally replicating expression plasmid for the HPV 16 early region. The initial analysis used pY71, containing the HPV 16 E5 and E7 ORFs; parts of E6, E1, E2, and E4 ORFs; and intact HPV 16 3′-end early sequences [Belaguli *et al.,* 1992] (Fig. 1). RNase protection assays of total cellular RNA with an HPV 16 nt 502–720 probe identified more slowly migrating AS E7 RNA from the pY71 construct (Fig. 2). The levels of AS and S RNA were comparable. In contrast, the pYSA plasmid containing no HPV 16 sequences 3′ of the E7 ORF in the same vector produced S RNA but no detectable AS RNA. Only a fraction of the S signal had the size of the expected fragment for pYSA, possibly due to nuclease. As expected, no AS RNA was present in CaSki cells, in which HPV 16 DNA is integrated and the early region 3′-end of viral DNA is absent (Figs. 1, 2) [Smits *et al.,* 1991].

To examine the possibility that nuclear posttranscriptional processing or nucleo-cytoplasmic transport affect the cellular distribution or translation of S or AS transcripts, cytoplasmic RNA was analysed (Fig. 3). S RNA and AS RNA were identified clearly in the cytoplasmic RNA from cells transfected with pY71. Cytoplasmic localization of the AS RNA was confirmed by *in situ* hybridisation analysis (data not shown). The level of cytoplasmic AS RNA was comparable to S RNA, as for the total cellular RNA (Figs. 2, 3). The results for

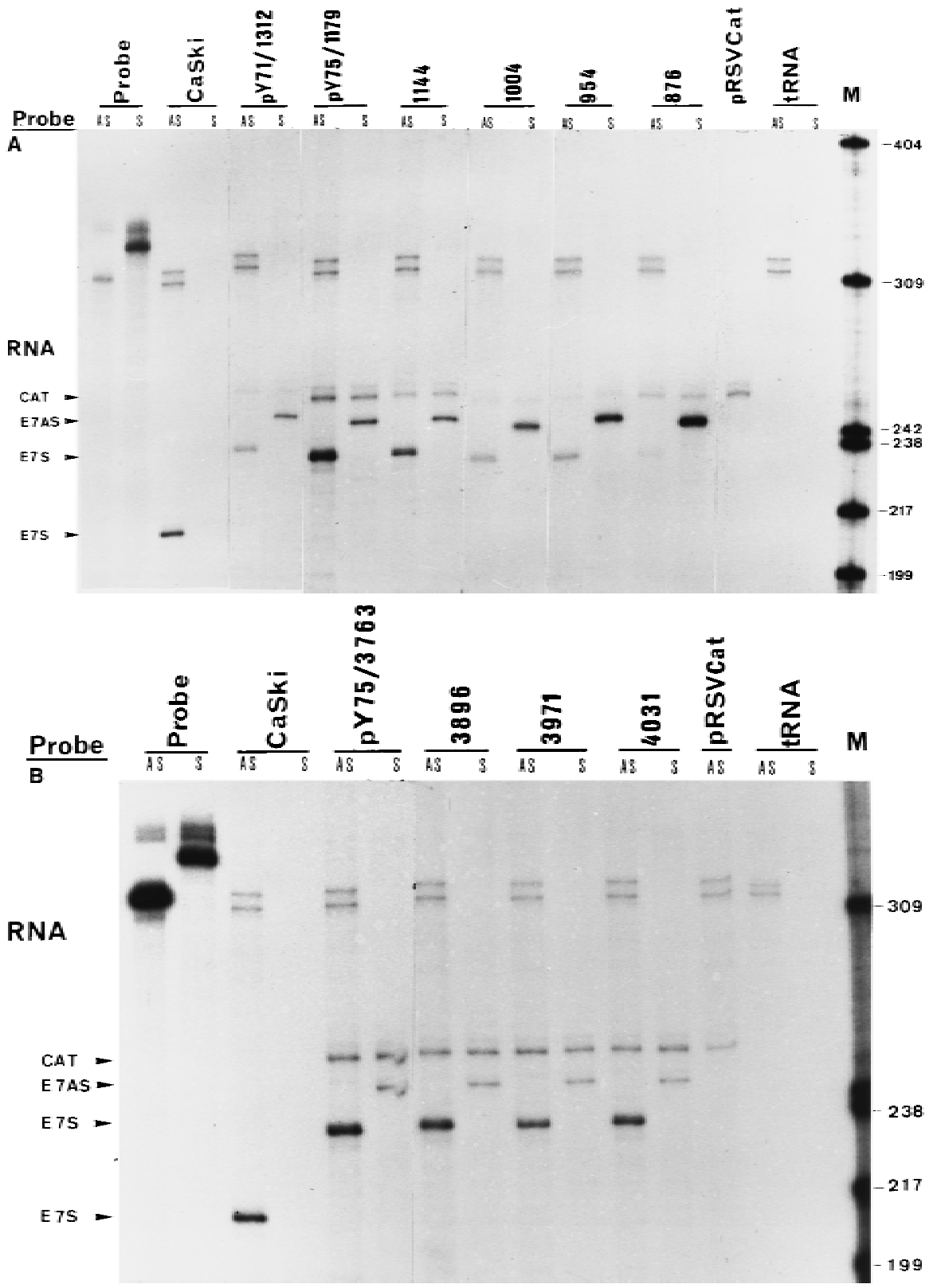

Fig. 4. HPV 16 antisense (AS) promoter location within nt 4031–4338. Total cellular RNA was analysed. Numbers above lanes are for the 5′ and 3′ series of deletions and represent (**A**) the 5′ nt to which deletions extend from the nt 1312–3763 deletion of pY71 and (**B**) the 3′ nt to which the deletions extend from the nt 1179–3763 deletion of pY75 (Fig. 1). Signals of internal control for transfection and gel-loading efficiency (CAT) and CAT-positive control (pRSVCat) lanes are indicated. Conditions and other labels are as for Figures 2 and 3.

pY75, which has a deletion of the sequences of the nt 1301 splice donor site (Fig. 1), confirmed the cytoplasmic presence of AS RNA. AS and S RNA levels were also similar for pY75 (Fig. 3).

Location of HPV 16 AS Promoter and Characterisation of RNA by Deletion Analysis

The promoter for the AS HPV 16 RNA found in previous studies was difficult to locate due to the low levels of the RNA or integrated status of the viral DNA [Higgins *et al.,* 1991; Vormwald-Dogan *et al.,* 1992]. For our episomal, high-level AS RNA, RNase protection assays of total cellular RNA for the longer deletion in pY75 also detected AS transcript (Figs. 1, 4). To examine which of the remaining HPV 16 sequences that were present in pY71 but not pYSA contained the AS promoter, progressively longer deletions were prepared and RNA was assayed (Fig. 4). The CAT gene was used as an internal cotransfection control for transfection efficiency and loading of the gels to allow comparison of HPV 16 transcript levels. Transfection efficiencies of the CAT control varied between the different RNA samples. However, both repeated RNase protection assays and pairs of assays for the CAT control for AS and S RNA for each RNA preparation gave consistent results. Also, the CAT control was used to compare results for the different preparations. None of the regions deleted between nt 876 and 3763 were essential for synthesis of AS RNA (Fig. 4A). An additional experiment showed that nt 570–876 also contained no AS promoter. Unilateral deletions further 3′ of that in Y75 and approaching the early poly(A) addition site were constructed and tested. Sequences from nt 3763 to 4031 were not essential because their deletion did not lead to a loss of AS RNA (Fig. 4B). However, the HPV 16 AS promoter was deleted for pY7SA, in which the remaining 307 nucleotides of HPV 16 3′ of E7 were absent, since no AS RNA was detected (Figs. 1, 2).

Two patterns were apparent for the level of AS RNA relative to S RNA. First, the AS RNA level increased and decreased relative to the S RNA level after deleting the nt 880 and 1301 splice donor sites, respectively. The effect of deleting the nt 1301 site can be seen by comparing results for pY75 with those for pY71. Normalizing with CAT RNA levels in the total cellular RNA, we observed a dramatically lower AS:S RNA ratio and AS RNA level (Fig. 4A). However, there was no apparent effect on the ratio or levels in cytoplasmic RNA (Fig. 3). The effect of deleting the nt 880 splice donor site can be seen by comparing the deletion constructs extending to nt 876 and 954. This deletion produced the opposite changes in the AS:S RNA ratio and the AS RNA level (Fig. 4A). Second, relative to the level of CAT control, the level of AS RNA obviously and progressively increased, while the level of S RNA remained unchanged; this was seen after deleting sequences from nt 1179 to 954. This deletes 5–33% of the distance between the probed E7 sequence and the 5′-end of AS RNA (Figs. 1, 4A).

HPV 16 AS RNA 5′ Boundary and Heterologous Function of AS Promoter

We wanted to examine the 5′ boundary of HPV 16 AS RNA, which was expected to define the 3′ boundary of sequences containing the AS promoter. Thus, an HPV 16 probe S to the 3′ early region mRNA was used for RNase protection assay of HPV 16 AS RNA of total cellular RNA. This probe contains sequences corresponding to nt 4107–4331, which overlap the HPV 16 early poly(A) addition site (Fig. 1). Therefore, the AS RNA probe was also useful for assaying processing of the HPV 16 3′-end of early S mRNA. However, the low level of 3′-end processing efficiency for the early region mRNA necessitated prolonged autoradiography (Fig. 5). Very low levels of AS RNA were detected, especially compared with S RNA. Smaller products than the probe were expected if the AS RNA was initiated 3′ of nt 4230. However, such products were also not found (Fig. 5). Together with the deletion analyses (Figs. 2, 4), these results indicate that the AS promoter is 3′ of nt 4030 and 5′ to 4230.

Next, a small HPV 16 promoter region was used to examine whether the AS promoter can function in a heterologous system. Thus, the nt 3978–4338 HPV 16 fragment was constructed into pSV2HPA2 (Fig. 1). The AS promoter in this plasmid was also functional for AS transcription of the heterologous CAT gene in RNase protection assays of total cellular RNA (Fig. 6). The S:AS RNA ratio was higher for pSV2HPA2 CAT than for pY71 E7 (Figs. 2, 4A, 6). This may be due to a factor such as greater stability of S CAT RNA compared with S E7 RNA, as suggested by the very high S CAT RNA level (Fig. 6). The level of CAT AS RNA for pSV2HPA2 containing the HPV 16 AS promoter greatly exceeded the levels of both the pSV2cat AS CAT RNA background signal, which was probably from promoter-like sequences of the vector, as well as the pRSV-β-Gal control GAL S RNA (Fig. 6). In addition, the levels of AS RNA for the homologous E7 and heterologous CAT genes were similar, relative to the control S RNA from the RSV expression vector (Figs. 4, 6). Therefore, the AS promoter contained within the 360 base pair fragment appeared to be functional and to not require additional *cis*-acting or *trans*-acting HPV 16 sequences.

AS-S dsRNA of HPV 16

The presence of AS and S RNA has been shown to produce dsRNA, which has important consequences for the expression of S RNA in other systems [reviewed by Nellen *et al.,* 1992]. Therefore, RNase protection was used to assess the formation of dsRNA. Standard RNase protection assays for total cellular RNA level were by denaturing the RNA, hybridising with probe, and then treating with RNase (Fig. 7, lanes labelled –). RNase protection assays for dsRNA level used additional RNase treatment to remove single-stranded RNA before denaturation (Fig. 7, lanes labeled +). Double-stranded HPV 16 E7 RNA was clearly observed

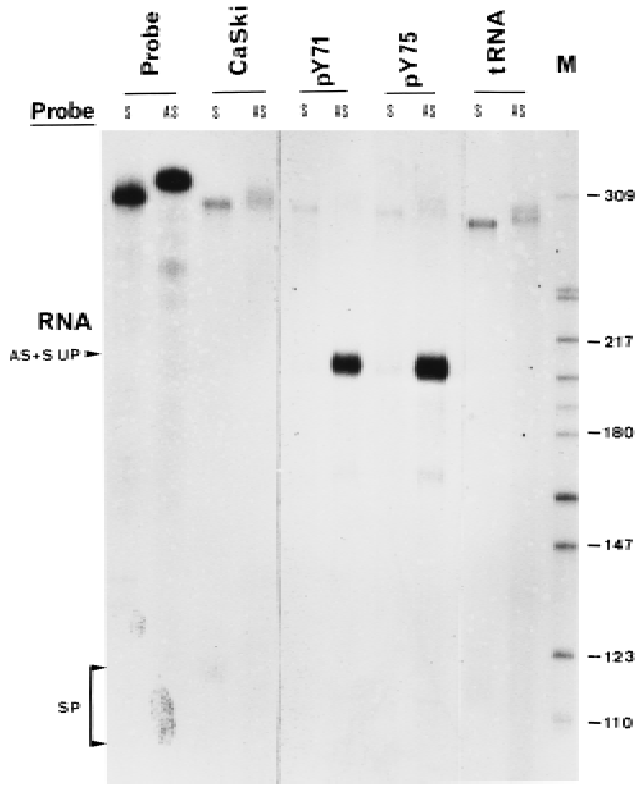

Fig. 5. Antisense (AS) promoter location and the effect of AS RNA on sense (S) RNA cleavage/poly(A) addition. Probe was for nt 4107–4311 assays using total cellular RNA. AS and S unprocessed (AS + S UP) and sense processed (SP) signals are indicated. Other conditions and labelling are as described in Figure 4. No signal was observed larger than 120 bases, locating the AS promoter 5′ of nt 4230. CaSki RNA does not contain HPV 16 13′-end sequences (Fig. 1). SP was a weak signal.

for the three constructs (Fig. 7A). The fraction of the total cellular S RNA that was in double-stranded form was large for pY71 and pY75 but small for pD7, which contains a much longer region between the AS promoter (Fig. 1, P-AS) and probe (Fig. 1, E7). This was consistent with results showing progressively reduced E7 AS RNA for the Figure 4A constructs, which contain longer early regions of HPV 16, since pD7 also has a much longer region between the AS promoter and the probe (Fig. 1). As expected, comparable levels of S and AS RNA were present in dsRNA (+ lanes) for all of the constructs (Fig. 7A). There was also no CaSki AS RNA or dsRNA, consistent with the deletion of the AS promoter of HPV 16 DNA in CaSki (Figs. 1, 7B) [Smits *et al.,* 1991].

Effects of HPV 16 dsRNA on Posttranscriptional Processing

To examine whether dsRNA is important posttranscriptionally, cleavage/poly(A) addition was assessed using an nt 4107–4311 probe. This probe was complementary to the HPV 16 poly(A) addition site at nt 4213 (Fig. 1, Poly[A]). The 3′-end cleavage of the mRNA of pY71 and pY75, which produced high levels of dsRNA (Fig. 7A), produced very low levels of RNA cleavage S products (Fig. 5, SP), especially for pY71. Longer autoradiography more clearly detected these products (data not shown).

We also examined the second important posttranscription process, splicing. A probe containing the nt 880 splice site sequences that are essential for HPV 16 E7 expression [Belaguli *et al.,* 1992] was used for assays of total cellular RNA. Mostly E7 unspliced (E7US) pY71 and pY75 RNA was seen. Splicing of pY71 E7 S (E7S) RNA was not detectable (Fig. 8A). Based on the total level of E7US and E7S RNA, the significantly greater level of single-stranded pY75 E7S RNA was spliced at a low level. The completely single-stranded CaSki E7 RNA (Fig. 7B) was efficiently spliced (Fig. 8A). Splicing was relatively efficient for the predominantly single-stranded RNA of pD7. However, splicing was also not detected for the high level of pY7, which was present as dsRNA (Fig. 8A, B). Splicing of S RNA was never detected for the double-stranded E7 RNA that was present at low levels for pD7 and at high levels for pY71 (Figs. 7A, 8B).

DISCUSSION

Only HPV 16 S promoters have been found and mapped to date. In this report, evidence for a novel HPV 16 promoter is provided. The episomally replicating vector system that was used is a model for HPV 16 DNA in benign and premalignant lesions containing nonintegrated DNA [zur Hausen and de Villiers, 1994]. More fundamentally, this system provided adequate HPV 16 E7 RNA levels for detection. The novel promoter was termed the AS promoter because of its orientation relative to the well-characterised P97 promoter [Smotkin *et al.,* 1989; Smotkin and Wettstein, 1986]. The AS promoter was within the nt 4030–4230 HPV 16 genome region. This was in contrast to the AS RNA for the integrated DNA of cervical cancers, which was thought to derive from a cellular promoter [Vormwald-Dogan *et al.,* 1992]. AS and S E7 RNA was found, as reported for the N-*myc* oncogene [Krystal *et al.,* 1990]. Our assays for the function of the HPV 16 AS promoter for the AS transcription of the heterologous CAT gene found a high AS CAT RNA level. The level was high relative to AS RNA from pSV2cat containing no HPV 16 sequences and to the pRSV-β-Gal internal control GAL S RNA. The HPV 16 AS promoter sequences used were from a specific 353 nucleotide fragment. This confirmed the deletion analysis, indicating that no other HPV 16 sequences were required for AS promoter function.

The properties of HPV 16 AS RNA may be explained by heterogeneous ends of the AS RNA. This heterogeneous nature was suggested by the inverse relationship between the level of AS RNA and the distance between the AS promoter and the E7 probe-homologous sequences in the deletion constructs. Although the deletions approaching the promoter region did not affect

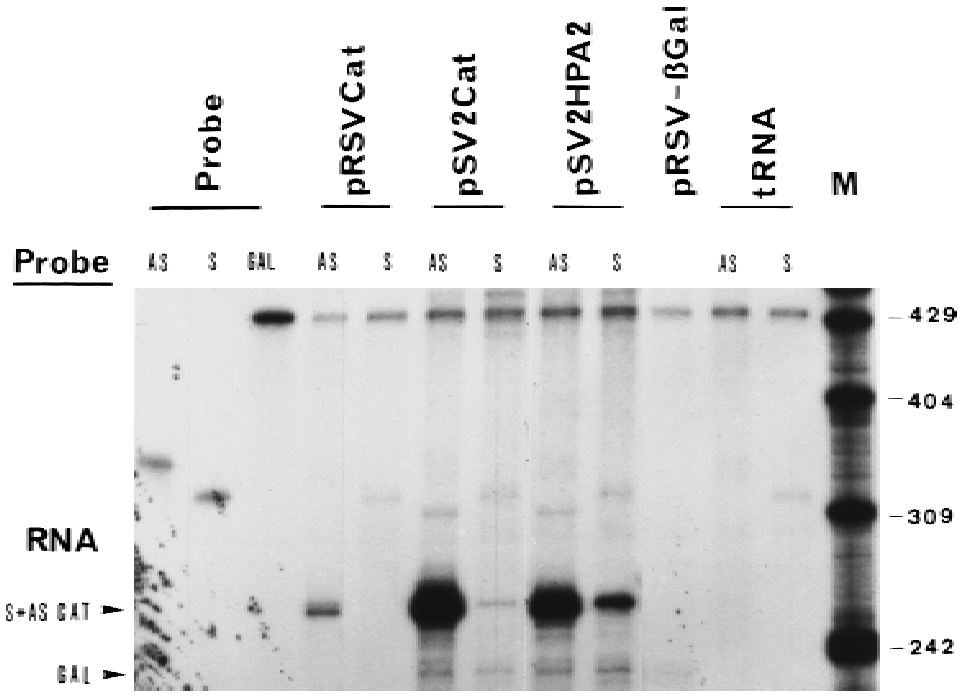

Fig. 6. Antisense (AS) expression of heterologous CAT gene. Lanes: CAT probes (AS and S); probe for β-galalactosidase (GAL); positive control pSV2cat (pSV2Cat); heterologous CAT gene-AS promoter plasmid pSV2HPA2 (pSV2HPA2); internal control pRSV-β-Gal (pRSV-βGal). On the left are indicated the signals for S and AS CAT (S + AS CAT) and for control β-galactosidase (GAL) RNA. Other conditions and labels are as in Figure 3.

the AS RNA level, some of the 5′-end initiation sites may have been deleted. The heterogeneous 3′-ends of AS transcripts are proposed to be the product of random termination of unstable AS RNA elongation complexes. The AS RNA would thus be short, explaining that its presence in the cytoplasm may be due to passive transfer. In a previous report, heterogeneous AS RNA ends for cervical carcinomas were observed and attributed to viral-cellular fusion transcripts [Vormwald-Dogan *et al.,* 1992]. Their failure to detect AS RNA by *in situ* hybridisation in premalignant lesions might be explained by our observations. The low level of AS E7 RNA observed for the complete HPV 16 early region would not be detected by their less sensitive *in situ* hybridisation assay. Our results also indicate that no AS transcripts would be long enough to contain L1 and noncoding region RNA, although some L1 AS RNA was observed previously [Vormwald-Dogan *et al.,* 1992].

dsRNA is important for regulating gene expression [reviewed by Nellen *et al.,* 1992]. The general effect of dsRNA is RNA instability. Consistently, we found that the level of AS RNA was much higher after deleting the essential nt 880 splice site, which led to greatly reduced S RNA levels. The opposite result for the deletion of the nt 1301 site may be due to a greater stability of E7 S mRNA spliced from the alternative nt 880 site (Fig 4), [Belaguli *et al.,* 1992]. The same might apply for the nt 226 E6 region splice site, which was important for providing the splicing function that was required for transformation and RNA accumulation in the absence of a functional nt 880 splice site [Belaguli *et al.,* 1995]. The dsRNA could also be differentially controlled in the various layers of infected stratifying squamous epithelial lesions by the level of dsRNA-specific unwindase [Wagner *et al.,* 1989]. Furthermore, viral dsRNA has been shown to interfere with normal cellular defense mechanisms against episomally replicating DNA virus [Matthews and Shenk, 1991]. This interference in the epithelial cells involved the tumor-suppressor gene product, dsRNA-dependent protein kinase [Kostura and Matthews, 1989; Wold *et al.,* 1994]. Therefore, the status of HPV 16 dsRNA might be important for induction of infectious and benign replication of viral and cellular DNA.

AS RNA acts at different posttranscriptional levels to interfere with normal cellular processes. AS RNA interferes with efficient splicing, reportedly by forming a double strand with S RNA [Munroe and Lazar, 1991]. In addition to the effect of dsRNA on splicing for pY71 and pY75, cleavage of the poly(A) addition site also appeared to be low. Interference with cleavage could be due to formation dsRNA poly(A) addition site or coupling of polyadenylation and splicing. Both of these processing events can be essential for mRNA stability and transport. Cytoplasmic AS RNA also may regulate translation of HPV 16 mRNA because regulation of gene expression by adenovirus dsRNA involves translation [Mathews and Shenk, 1991]. Moreover, artificial AS constructs inhibited expression of HPV 11 and 16

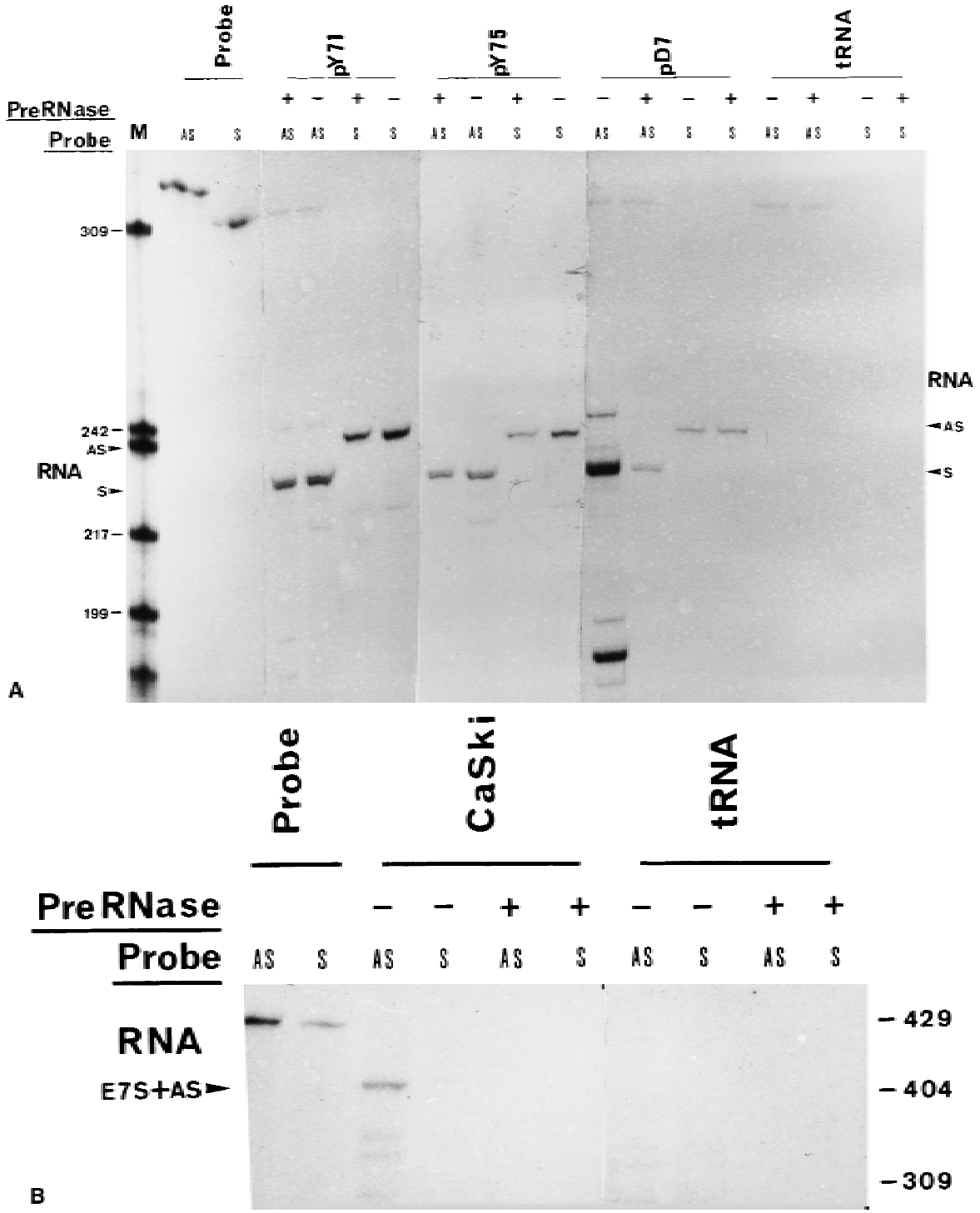

Fig. 7. Double-stranded (ds) HPV 16 antisense-sense (AS-S) RNA and the effect of deletions. RNase protection assays used total cellular RNA and RNase treatment before and after hybridisation (PreRNase and + for lanes) to detect dsRNA, and only after hybridisation (PreRNase and – for lanes) to detect signals in total cellular RNA. Other conditions and labels are as in Figures 1–4. **A:** Probe was for nt 502–720. **B:** Probe was for nt 792–1179. The order of the four lanes for each RNA preparation is different from the order for (A).

by interfering with translation or transcription [Cowsert *et al.,* 1993; Tan *et al.,* 1994].

HPV mRNA expression is closely coupled to host epithelial cell differentiation [Crum *et al.,* 1988; Durst *et al.,* 1992]. This may also apply to AS RNA expression. Differential HPV regulation by AS RNA could influence the growth properties of differentiating host cells. We found early AS RNA in HPV 16-immortalised cells

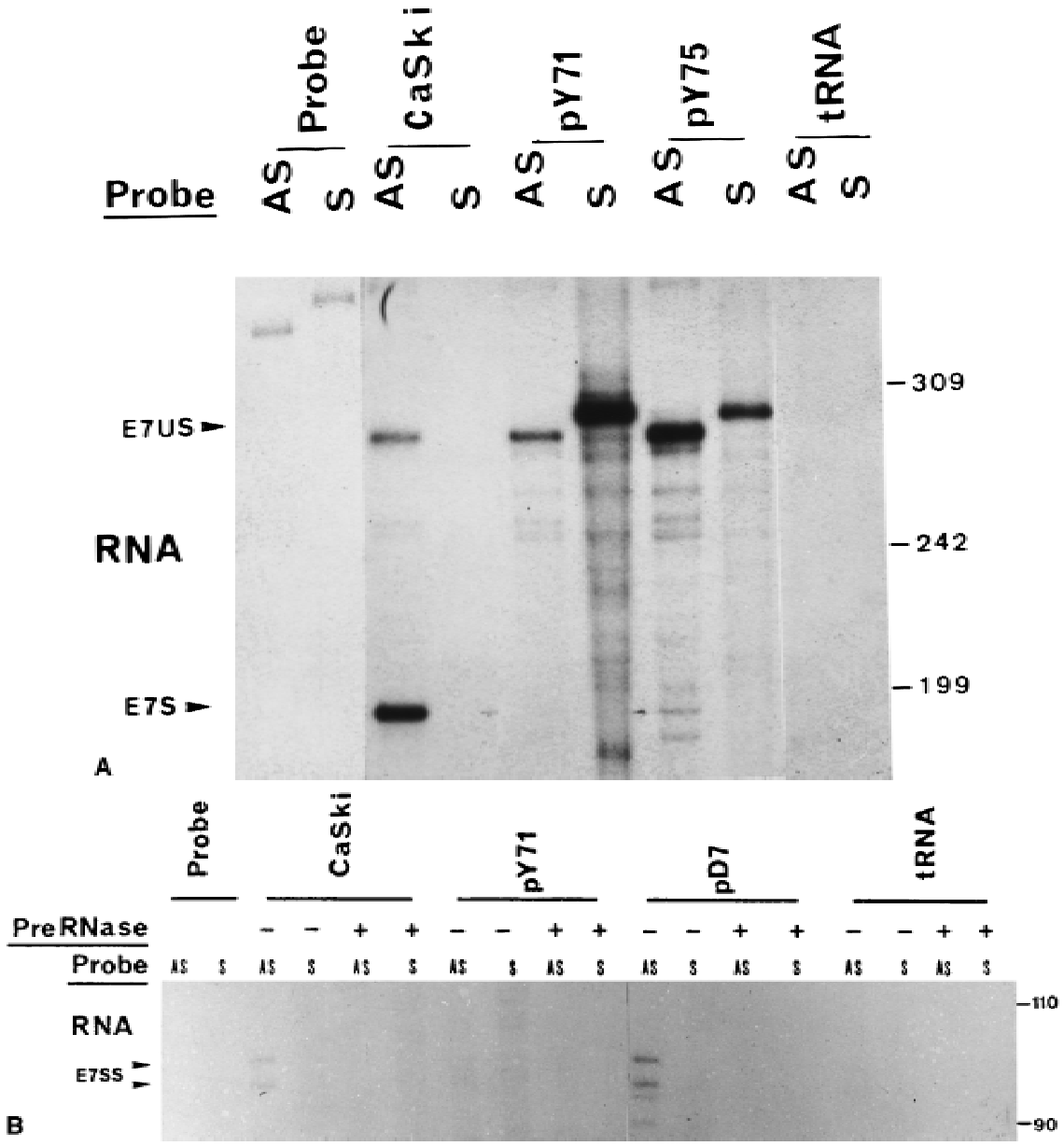

Fig. 8. Effect on splicing of HPV 16 antisense (AS) RNA and of deletions. **A:** Probe for total cellular RNA was for nt 720–955. E7 unspliced (E7US) and E7 spliced (E7S) RNA are indicated on the left. Other labelling and conditions are as for Figure 3. **B:** Probe was for nt 792–1179. E7 spliced sense (E7SS) RNA products are indicated. Other labelling and conditions are as in Figure 7.

[Tsutsumi *et al.,* 1994]. However, the level was low, and natural infections or transfections provide little HPV 16 RNA. Expression of high levels of AS E6-E7 RNA inhibited the growth of HPV 18-containing epithelial cells [von Knebel-Doeberitz *et al.,* 1992]. Thus, AS RNA may act indirectly or directly in a posttranscriptional regulation mechanism of HPV 16 gene expression and cellular growth responses. Alternately, the mechanism of oncogenesis by HPV 16 may involve the AS promoter and viral or cell gene expression. Similar to the pY7 series plasmids, 3′ early region sequences of integrated HPV 16 DNA are often deleted from cervical carcinomas and their cell lines. This deregulation could be due to the absence of cell-specific regulation of the HPV AS promoter. The E2 transactivator and E5 signal transduction pathway factor sometimes are reported to be intact. However, integration occurring during oncogenesis can remove or translocate the AS promoter. The AS promoter sequences were deleted, and our results showed no AS RNA for CaSki [Smits *et al.,* 1991]; (Fig. 1). Therefore, deregulated HPV 16 expression might result from loss of the AS promoter by integration 5′ to the AS promoter. Alternately, AS promoter-adjacent cellular sequences may deregulate the promoter.

Cellular gene expression may also be involved, based on several findings. First, integration of HPV has been

associated with deregulated expression of oncogenically important cellular genes [Durst *et al.,* 1987]. Second, oncogenic expression of one such gene, c-*myc,* was associated with deregulated AS c-*myc* RNA [Spicer and Sonenshein, 1992]. Third, we show that the HPV 16 AS promoter was functional for expression of homologous sequences and heterologous CAT gene expression. Therefore, an additional mechanism for deregulating expression of genes that are involved in oncogenesis could involve altered expression of important cellular genes from the HPV 16 AS promoter following integration. An example may be CaSki, for which a simple insertion of HPV 16 DNA could have inserted the AS promoter into cellular sequences which code for an oncogene or tumor-suppressor gene. Consistent with this hypothesis, the integrated HPV 16 of CaSki used the cellular sequences adjacent to the HPV 16 integration site for efficient cleavage/poly(A) addition function [Smotkin and Wettstein, 1986; Smits *et al.,* 1991]. This suggests that integration of HPV 16 also oriented the AS promoter upstream of the poly(A) addition site of a functional cellular gene. In this orientation, AS RNA for this possibly important gene could be abnormally produced, similar to our observations for the heterologous CAT gene. Thus, HPV 16 integration could deregulate HPV 16 expression from the AS promoter by deleting it or by inserting it in front of cellular regulatory sequences. Alternately, AS RNA could deregulate cellular gene expression via the production of AS cellular RNA. The deregulation of both is important for oncogenesis [zur Hausen and de Villiers, 1994].

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